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Identification and Purity Test of Melon Cultivars and F_1 Hybrids Using Fluidigm-based SNP Markers

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Abstract

In melon (*Cucumis melo* L.), the grow-out test (GOT) has been traditionally used as a genetic purity test. However, this method is time-consuming, space-demanding, and associated with ambiguous classification of the genotypes. Molecular markers have proved to be an efficient tool in genotyping analyses. In the present study, a total of 96 genome-wide single nucleotide polymorphism (SNP) markers differentiated 85 melon F_1 hybrid plants from their parental lines and six other PT melon breeding lines via high-throughput Fluidigm genotyping. Of these, 39 SNP markers showed polymorphism between the parents. Additionally, SNP analysis and population structure analysis showed that several F_1 hybrid plants were associated with outcrossing during the breeding program. Unweighted pair group method with arithmetic average (UPGMA) analysis revealed that most of the contaminated plants were closely sub-grouped with the 7_PT1 breeding line, suggesting possible outcrossing with 7_PT1. By combining with simple DNA extraction, the Fluidigm-based SNP marker analysis proved to be a simple and effective approach for the genetic purity analysis of F_1 hybrids and melon cultivars.

Additional key words: F_1 hybrids and breeding lines, Fluidigm analysis, genetic purity analysis, melon, single nucleotide polymorphism (SNP)

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Introduction

Cucumis melo is an important vegetable crop and includes several subspecies, where introgression from unknown germplasms continues to be a critical problem in breeding programs. In recent years, melon F_1 hybrids were developed and released into the seed market by various seed companies (Nguyen et al., 2019; Kishor et al., 2020). The success of any breeding program mostly depends on an adequate supply of genetically pure hybrid seeds in the seed market. Therefore, seed companies require genetic purity tests, which are verified based on the success rate of cross-pollination and the number of plants with self-pollination, assuring good quality seeds.

In the past, genetic purity testing was performed by the grow-out test (GOT), which involves characterizing representative samples of F_1 hybrid seeds into true hybrid seeds or off-types based on

several morphological characteristics at various stages of plant growth (Pattanaik et al., 2018). However, this method is associated with many limitations. It is time-consuming, space-demanding, and yields ambiguous classification of the genotypes. Besides that, environmental influences on morphological characteristics also make it difficult to obtain accurate morphological data. In addition to the GOT, various biochemical methods, such as isozyme analysis followed by electrophoresis, have been used for the genetic purity testing of F_1 hybrids (Markova et al., 2003; Jadhav and Achar, 2016). These techniques have advantages but are associated with limited polymorphism and environmental sensitivity.

Molecular markers are considered to be an efficient tool for genetic purity analysis due to their simple, fast, and accurate applications (Cheng-Xiang et al., 2005; Luan et al., 2010; An et al., 2010; Bae et al., 2015). Simple sequence repeats (SSRs) have become the most preferred marker for genetic purity analysis in melons (Cheng-Xiang et al., 2005; Luan et al., 2010). However, regions flanking SSRs could contain insertions or deletions (INDELs) or other SSR events (Bang and Chung, 2015) and the origin of the total length variation of the SSR markers should be confirmed by sequencing before F_1 genetic purity analysis. Therefore, employing single nucleotide polymorphism (SNP)-based markers for F_1 genetic purity analysis can overcome the limitations of SSR markers. SNPs are the most ideal markers because of their high abundance, even distribution, and strong marker-trait associations (Hayward et al., 2012), thus capturing importance for genetic diversity studies (Heo et al., 2017; Li et al., 2019).

The present study assessed the genetic purity of hybrid seeds of various commercial melons using genome-wide SNP markers developed by our group (Kishor et al., 2020). This study aimed to validate these genome-wide SNP markers to assess the genetic purity of F_1s and PT breeding lines in melons.

Materials and Methods

Plant Materials and DNA Isolation

Eight PT breeding lines, 7_PT1, 10_PT10, 35-1_PT35, 46-1_PT46, 2H104_PT104, 2H106_PT106, PT1_male, and PT1_female, and 85 F_1 hybrid plants derived from the cross between PT1_male and PT1_female were used in this study. All the eight PT melon breeding lines and 85 F_1 hybrid plants were developed at Changchun Jongmyo Co., Ltd., Chilkog, Republic of Korea. The young leaves of 85 F_1 hybrid plants, their parents, and other PT melon breeding lines were obtained and subjected to DNA extraction. Total genomic DNA (gDNA) was isolated from leaf tissue using the SDS procedure with slight modifications (Kim et al., 1997). The quality and quantity of DNA were determined by measuring the O.D. at 260/280 nm using a DS-11 spectrophotometer (Denovix Inc., DE, USA) followed by 1.2% gel electrophoresis.

SNP Markers and Fluidigm Genotyping

A total of 96 genome-wide SNP markers were obtained from our recent study (Kishor et al., 2020) and used for the genetic purity analysis of 85 F_1 hybrid melon plants and their parents (Table 1).

Genotyping was conducted using the Fluidigm Juno system (Fluidigm Corporation, CA, USA). The step involving pre-amplification was performed using both specific-target amplification (STA) and locus-specific primers (LSP), followed by dilution of the pre-amplified products with distilled water. PCR amplification was performed using a set of allele-specific primers (ASP). End-point reads were detected using a Biomark EP1 Reader (Fluidigm Corporation, CA,

USA). The SNP calling was performed according to the Fluidigm Juno protocol using the Fluidigm SNP Genotyping Analysis software.

Table 1. The selected 96 SNPs for genetic purity analysis

S. No	SNP Id.	Position	Chromosome	Allele	Polymorphism in PT1 parents
1	M1_884520	884520	1	G/T	
2	M1_975872	975872	1	A/G	
3	M1_1066595	1066595	1	T/A	
4	M1_1173173	1173173	1	C/T	
5	M1_1296246	1296246	1	A/G	
6	M1_1462130	1462130	1	T/G	
7	M1_1978773	1978773	1	G/T	
8	M1_2112637	2112637	1	C/T	
9	M1_3013958	3013958	1	A/G	Yes
10	M1_4104877	4104877	1	G/A	
11	M1_4325219	4325219	1	G/A	
12	M1_6614453	6614453	1	A/T	Yes
13	M1_7977218	7977218	1	T/C	
14	M1_8036666	8036666	1	T/A	
15	M1_8793338	8793338	1	G/A	
16	M1_8819586	8819586	1	C/T	
17	M1_8896747	8896747	1	A/G	
18	M1_9274229	9274229	1	C/T	Yes
19	M1_14858123	14858123	1	G/A	Yes
20	M1_14897464	14897464	1	A/G	Yes
22	M1_30163614	30163614	1	T/C	
23	M1_30481228	30481228	1	T/G	
24	M1_30584172	30584172	1	T/C	
25	M1_31094861	31094861	1	T/C	
26	M1_31143403	31143403	1	G/T	
27	M1_31442835	31442835	1	G/A	
28	M1_31869094	31869094	1	T/G	
29	M1_31872750	31872750	1	C/T	
31	M2_1591748	1591748	2	G/T	
32	M2_3245603	3245603	2	A/G	
33	M2_15448280	15448280	2	A/T	
34	M2_15448306	15448306	2	G/A	
35	M2_15556647	15556647	2	A/G	
36	M2_22107827	22107827	2	T/A	
37	M2_25707021	25707021	2	C/T	Yes
30	M2_34458684	34458684	2	G/A	Yes
38	M3_86815	86815	3	T/C	Yes
39	M3_2128779	2128779	3	A/T	Yes
40	M3_2499868	2499868	3	G/T	Yes
41	M3_2792358	2792358	3	C/T	Yes
42	M3_3417026	3417026	3	C/T	
43	M3_5526710	5526710	3	G/C	Yes
44	M3_5568974	5568974	3	A/T	Yes
45	M3_5829995	5829995	3	A/C	
46	M3_6312154	6312154	3	G/A	Yes
47	M3_23854097	23854097	3	G/A	

Table 1. The selected 96 SNPs for genetic purity analysis (Continued)

S. No	SNP Id.	Position	Chromosome	Allele	Polymorphism in PT1 parents
48	M3_24188878	24188878	3	C/T	
49	M3_28982354	28982354	3	G/A	Yes
50	M3_28997081	28997081	3	C/G	Yes
51	M4_1414734	1414734	4	C/T	Yes
52	M4_10188263	10188263	4	G/T	Yes
53	M4_15809728	15809728	4	G/A	
54	M4_17312594	17312594	4	C/G	
55	M4_18961613	18961613	4	C/T	
56	M10_1291758	1291758	10	G/A	Yes
57	M10_1537667	1537667	10	G/A	
58	M10_1537793	1537793	10	G/A	
59	M10_4361263	4361263	10	A/G	
60	M10_4736855	4736855	10	G/A	Yes
21	M10_18318803	18318803	10	A/G	
61	M10_18383943	18383943	10	T/C	
62	M10_18384386	18384386	10	A/T	
63	M10_19109584	19109584	10	A/G	
64	M10_19172671	19172671	10	T/C	
65	M10_19174142	19174142	10	C/T	
66	M10_19372215	19372215	10	G/A	Yes
67	M10_21781267	21781267	10	T/G	
68	M11_3581140	3581140	11	C/G	Yes
69	M11_18095430	18095430	11	A/C	Yes
70	M11_27693096	27693096	11	G/A	Yes
71	M12_1557777	1557777	12	A/T	Yes
72	M12_2513136	2513136	12	C/A	
73	M12_6385196	6385196	12	G/A	Yes
74	M12_9676532	9676532	12	G/A	Yes
75	M12_9676722	9676722	12	G/A	Yes
76	M12_9699659	9699659	12	G/C	Yes
77	M12_9699917	9699917	12	T/C	Yes
78	M12_9700013	9700013	12	A/C	Yes
79	M12_11236131	11236131	12	G/C	Yes
80	M12_11374398	11374398	12	T/C	Yes
81	M12_11422892	11422892	12	G/C	
82	M12_13049747	13049747	12	A/G	Yes
83	M12_15922782	15922782	12	A/G	Yes
84	M12_17012579	17012579	12	G/A	
85	M12_18942955	18942955	12	A/G	
86	M12_19163631	19163631	12	A/C	
87	M12_20876389	20876389	12	G/A	Yes
88	M12_21687813	21687813	12	A/T	Yes
89	M12_22072754	22072754	12	T/C	
90	M12_22234004	22234004	12	G/T	
91	M12_22620200	22620200	12	C/A	Yes
92	M12_22620201	22620201	12	C/A	
93	M12_22647941	22647941	12	G/C	Yes
94	M12_22820505	22820505	12	C/T	
95	M12_22906257	22906257	12	C/T	
96	M12_23157448	23157448	12	C/T	

Genetic Classification

Contamination of the 85 F_1 hybrid plants with the genetic material of other varieties or species was determined using polymorphic SNP markers in the STRUCTURE 2.3.4 program (Falush et al., 2003). The burn-in period was performed using 100,000 iterations, followed by 100,000 Markov chain Monte Carlo (MCMC) iterations per run. The number of genetically distinct populations (*K*) was adjusted from 1 to 10, and the model was repeated three times for each *K*. The best *K* value was estimated based on the delta *K* (ΔK) value using STRUCTURE HARVESTER (Earl and von Holdt, 2012). Similarly, an unweighted pair group method with arithmetic average (UPGMA) tree was constructed using Cavalli-Sforza and Edwards' (1967) genetic distance method in PowerMarker V3.25 (Liu and Muse, 2005). The UPGMA tree was constructed using the SNP assay results of 95 commercial melon cultivars (M1 to M95) from our previous study (Kishor et al., 2020), and the SNP assay results of the present study.

Results

SNP Marker Analysis

From the 96 SNP marker assay in the 85 F_1 hybrid plants, their parents, and other PT melon breeding lines (Fig. 1), 89 SNP markers (92.70%) had successful DNA amplification, of which 39 SNP (43.82%) markers showed stable

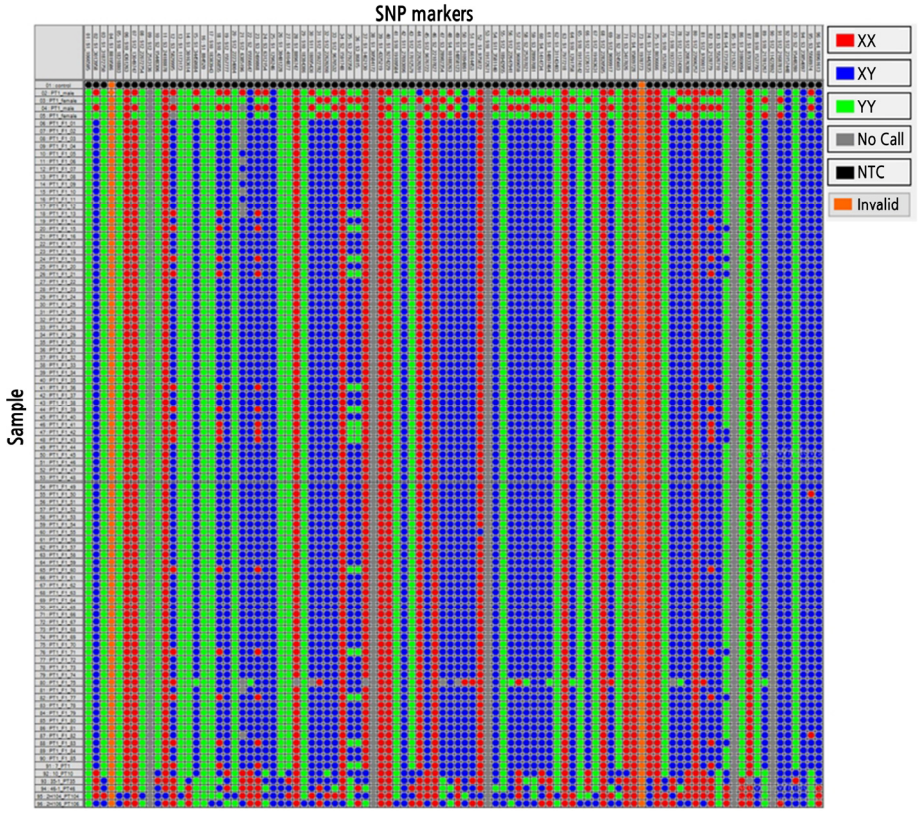

Fig. 1. Image showing amplification success of SNP analysis in 85 PT_1 F1 hybrid plants, their parents, and six other PT melon breeding lines. SNP calling was chatacterized as XX and YY for homozygotes, XY for heterozygotes, no calls, no template control (NTC), and invalid data.

polymorphisms between PT1_male and PT1_female (Table 1). Therefore, these 39 polymorphic SNP markers were used to distinguish the F_1 hybrid plants and their parents in the present study (Table 1). In contrast, the other 50 SNP markers could not amplify the DNA in Fluidigm genotyping but displayed no calls, monomorphism, or heterozygosity in the F_1 hybrid plants and their parents.

Based on the six markers, M3_5822995, M10_4736855, M3_2738358, M3_86815, M3_2128779, and M4_17312594, F_1 hybrid plant numbers PT1 F_1 13, PT1 F_1 15, PT1 F_1 19, PT1 F_1 21, PT1 F_1 36, PT1 F_1 39, PT1 F_1 41, PT1 F_1 42, PT1 F_1 43, PT1 F_1 60, PT1 F_1 71, PT1 F_1 77, and PT1 F_1 83 showed amplification errors in the SNP assay. Additionally, F_1 hybrid plant numbers PT1 F_1 50 and PT1 F_1 82 showed amplification errors in the M3_5568974 SNP marker only. These results suggest that amplification errors might be associated with outcrossing. Similarly, a genotyping error was also observed in F_1 hybrid plant number PT1 F_1 55 for the M1_975872 monomorphic SNP marker, which could be due to the amplification of a non-target site. In contrast, all 89 SNP markers successfully discriminated genotypes such as 7_PT1, 10_PT10, 35-1_PT35, 46-1_PT46, 2H104_PT104, and 2H106_PT106 in the SNP assay.

Genetic Purity in F_1 Hybrid Plants

Contamination with other genetic material of other varieties or species was determined by using 39 polymorphic SNP markers in the model-based STRUCTURE program; this assumes many populations among 85 PT1 F_1 hybrid plants. The delta *K* value was maximum at *K*=2 (Fig. S1). The individuals under the different populations with scores of more than 0.80 were classified as pure and scores of less than 0.80 as admixture plants. The results showed that F_1 hybrid plant numbers PT1 F_1 13, PT1 F_1 15, PT1 F_1 19, PT1 F_1 21, PT1 F_1 36, PT1 F_1 39, PT1 F_1 41, PT1 F_1 42, PT1 F_1 43, PT1 F_1 60, PT1 F_1 71, PT1 F_1 77, and PT1 F_1 83 were grouped into a separate cluster (Fig. 2), suggesting the contamination with other genetic material of other varieties due to outcrossing. Additionally, F_1 hybrid plant number PT1 F_1 75 showed admixed genetic material. Hence, these plants should not be considered for the selection process due to outcrossing.

Genetic Relationship Analysis

A UPGMA tree was constructed based on the SNP assay results of 96 SNP markers in 95 commercial melon cultivars (Kishor et al., 2020), and SNP assay results of the present study, which included 7_PT1, 10_PT10, 35-1_PT35, 46-1_PT46, 2H104_PT104, 2H106_PT106, PT1_males, and PT1_females, F_1 hybrid plant numbers PT1 F_1 01, PT1 F_1

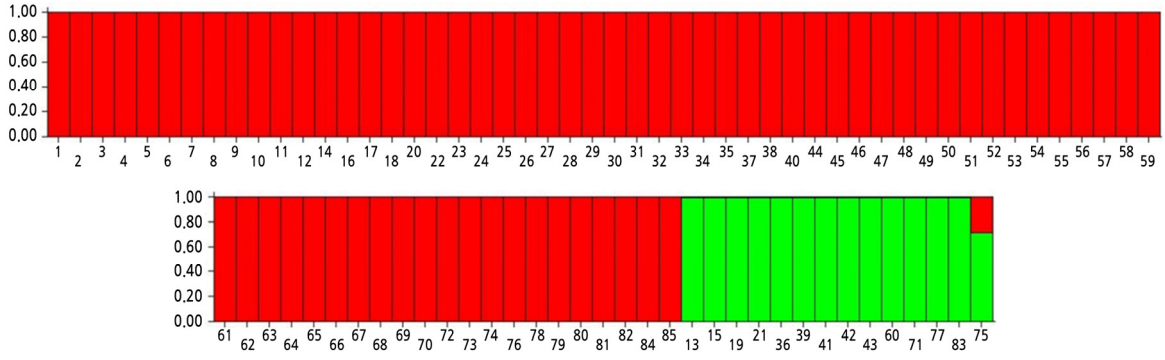

Fig. 2. Population structure analysis of 85 PT_1 F1 hybrid plants. Numbers 1 to 85 indicate the 85 PT1 F_1 hybrid plants in numerical order.

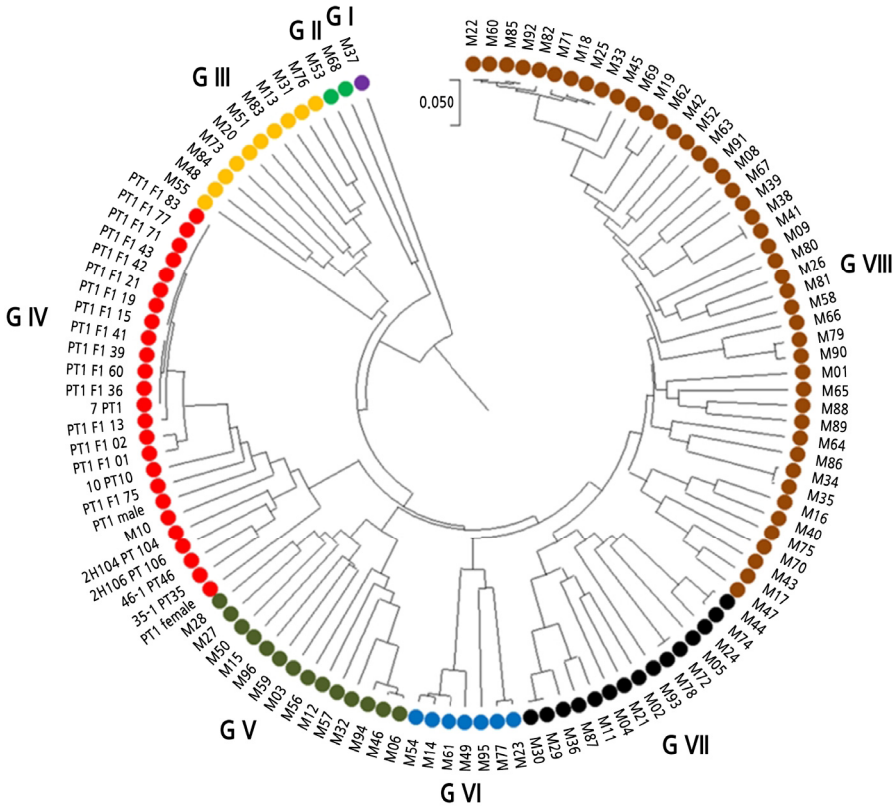

Fig. 3. UPGMA dendrogram based on Cavalli-Sforza and Edwards' (1967) genetic distance using 96 SNP markers in 119 samples consisting of 95 melon cultivars (M1 to M95) from our previous study (Kishor et al., 2020), PT1 F_1 hybrid plants, and PT melon breeding lines. All eight distinct groups were assigned different colors.

02, PT1 F_1 13, PT1 F_1 15, PT1 F_1 19, PT1 F_1 21, PT1 F_1 36, PT1 F_1 39, PT1 F_1 41, PT1 F_1 42, PT1 F_1 43, PT1 F_1 60, PT1 F_1 71, PT1 F_1 77, and PT1 F_1 75, classified into eight distinct groups (Fig. 3). Most of the melon cultivars were distributed in group VIII, followed by group IV and group VII based on the 96 SNP markers. However, M37 deviated as a separate group I.

All the PT1 melon breeding lines and F_1 hybrid plant numbers were grouped with M10 in group IV. Interestingly, all the PT1 melon breeding lines, F_1 hybrid plants, and M10 were developed at the Changchun Jongmyo Co., Ltd, Company, Chilkog, Republic of Korea. Additionally, F_1 hybrid plant numbers PT1 F_1 13, PT1 F_1 15, PT1 F_1 19, PT1 F_1 21, PT1 F_1 36, PT1 F_1 39, PT1 F_1 41, PT1 F_1 42, PT1 F_1 43, PT1 F_1 60, PT1 F_1 71, and PT1 F_1 77 were closely sub-grouped with the 7_PT1 melon breeding line in group IV. However, F_1 hybrid plant number PT1 F_1 75 deviated as a separate sub-group in group IV.

Discussion

The genetic purity of hybrids and cultivars is of great importance for the success of any breeding program. In the present study, 85 F_1 hybrid plants and eight PT melon breeding lines were evaluated using genome-wide SNP markers to assess genetic purity.

Among the various DNA-based markers, SSR markers are predominantly used for genetic purity analysis of melons (Cheng-Xiang et al., 2005; Luan et al., 2010). However, these markers are associated with INDELs or other SSR events within its flanking region (Bang and Chung, 2015). Recently, Next-generation Sequencing (NGS) technology gaining importance to generate large number of SNPs and used to develop SNP markers (Jung et al., 2020; Kishor et al., 2020). Similarly, previous studies were also reported handful of high-resolution melting (HRM)-based SNP markers for genetic purity analysis of F_1 hybrids (An et al., 2010) and powdery mildew race 5-specific SNP markers in *Cucumis melo* (Howlader et al., 2020). Presently, however, there are very limited numbers of these SNP markers for melons. In the latest study, we reported genome-wide SNP markers via genotyping-by-sequencing (GBS) in melons (Kishor et al., 2020). This study revealed that numerous high-quality SNP markers were distributed across the 12 chromosomes in the melon genome.

Here, we used 96 high-quality SNP markers from our previous study (Kishor et al., 2020) and performed genetic purity analysis of F_1 hybrids and PT breeding lines in melon via Fluidigm SNP assays. The results indicated a 92.70% success rate of DNA amplification in both the 85 F_1 hybrid plants and the eight PT melon breeding lines. Although a total of 96 SNP markers were screened, 39 SNP (43.82%) markers showed stable polymorphisms between the PT1_male and the PT1_female. Such a result was associated with highly similar genetic backgrounds between the parents, possibly due to the relatively narrow genetic base in the crop plants (Pattanaik et al., 2018).

In a breeding program, seeds can become admixed due to several reasons, such as outcrossing, pollen shedders, and physical mixtures (Pattanaik et al., 2018). Therefore, providing genetically pure hybrid seed is very important for commercially successful hybrids. A recent study reported that admixed cultivars could be classified using population structure analysis in melons (Kishor et al., 2020).

In the present study, we identified several F_1 hybrid plants associated with contamination due to outcrossing based on SNP marker analysis and population structure analysis. Additionally, the SNP analysis and population structure analysis results identifying the genetic purity of F_1 hybrid plants were consistent with each other. Hence, these contaminated plants are not recommended for further selection processes. Further, UPGMA analysis revealed that most of the contaminated plants were closely sub-grouped with 7_PT1, suggesting possible outcrossing with the 7_PT1 melon breeding line.

Together with DNA extraction, the Fluidigm-based SNP marker analysis presents a simple and effective approach for quality testing of melon hybrids and breeding lines. Additionally, SNP marker technology could identify genetic similarities and differences by comparing melon PT breeding lines with commercial or registered melon cultivars. Therefore, these SNP markers can help breeders protect the plant proprietary rights of new cultivars or hybrids through genetic purity testing of the melons.

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